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14. ABSTRACT Mutations have been recently identified in the EphB2 receptor gene in prostate cancer suggesting that EphB2, a member of the large Eph receptor tyrosine kinase family, is a tumor suppressor in prostate cancer. Consistent with a tumor suppressor activity, we found that EphB2 is more highly expressed in non-transformed BPH-1 prostate epithelial cells than in several prostate cancer cell lines. Furthermore, EphB2 expression was rapidly lost in stably transfected DU145 prostate cancer cells, suggesting that EphB2 inhibits cell growth and/or survival. We plan to further examine the effects of EphB2 signaling on the behavior of cancer cells in tissue culture and on prostate cancer progression in a mouse xenograft model. We will also examine whether other Eph receptors that we have detected in prostate cancer cells have effects similar to EphB2. The information obtained from these studies will help guide the design of appropriate treatment strategies and determine if prostate cancers should be screened for Eph receptor and ephrin ligand expression for prognostic purposes.					
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Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusions.....	8
References.....	9
Figures.....	11
Appendices.....	none

INTRODUCTION

Loss of function of tumor suppressor genes and increased function of tumor-promoting genes are critical steps in the development and progression of cancer. It is therefore important to identify these genes and understand how they affect cancer progression in order to develop new treatments. Previous work provided intriguing clues suggesting that the EphB2 receptor, a member of the large Eph receptor tyrosine kinase family, is a tumor suppressor in prostate cancer. In particular, inactivating mutations in the EphB2 gene were identified in clinical prostate cancer samples, and not in normal tissue, and forced expression of EphB2 was shown to suppress the growth of cultured prostate cancer cells that lack EphB2 expression (Huusko et al., 2004). Furthermore, a nonsense mutation in the EphB2 gene has been recently associated with prostate cancer risk in African American men with a positive family history (Kittles et al., 2006). Several mechanisms of EphB2 inactivation in colorectal tumors have also been recently reported, supporting the hypothesis that EphB2 functions as a tumor suppressor (Alazzouzi et al., 2005). However, conclusive evidence for a tumor suppressor role of EphB2 is lacking and the signaling mechanisms that may underlie such a role are completely unknown.

Our work with another Eph receptor in breast cancer supports that idea that activation of Eph receptor signaling pathways by their ligands, called ephrins, can inhibit the malignant properties of cancer cells (Noren et al., 2006). Our hypothesis is that signaling pathways activated by EphB2, and possibly other Eph receptors, negatively regulate the malignant properties of prostate cancer cells. Importantly, since submission of this application several reports have appeared in the literature highlighting the potentially critical role of different Eph receptors in the pathogenesis of prostate cancer (Fox and Kandpal, 2006; Lee et al., 2005; Xia et al., 2005).

BODY

Aim 1. Determine whether the growth-suppressing activity of EphB2 in prostate cancer cells depends on its activation by ephrin ligands

Task 1. Screen prostate cancer cell lines by immunoblotting and immunoprecipitation to determine expression and activation of selected Eph receptors and ephrins.

We compared the levels of expression of several Eph receptors and ephrin ligands in non-transformed BPH-1 prostate epithelial cells and in three well characterized prostate cancer cell lines (DU145, PC3 and LNCaP) (Fig. 1A). For comparison, we also examined Cos7 cells and mouse brain because their patterns of Eph receptor and ephrin expression are known. We found that EphB2 expression was much lower in the prostate cancer cell line than in the non-transformed BPH-1 cells, consistent with a downregulation of EphB2 expression during prostate cancer progression. The levels of EphB2 were as high in the BPH-1 cells as they were in Cos7 cells and P16 mouse brain, which are known to express high levels of EphB2. These data are consistent with a recent report examining EphB2 mRNA levels in transformed versus non-transformed prostate epithelial cell lines (Fox et al., 2006). EphB2 also appeared to have a smaller size in the LNCaP cells (Fig. 1A, B), suggesting the presence of an alternatively spliced or mutated form in these cells. We did not detect EphB2 in the DU145 cell line, as expected (Huusko et al., 2004). We detected low levels of EphB2 tyrosine phosphorylation in the PC3 and LNCaP cell lines, indicating a low level of activation. This result is also consistent with our hypothesis that EphB2 activation would inhibit tumorigenesis.

When examining expression of the EphB2 ligands, ephrin-B1, ephrin-B2 and ephrin-B3, we only detected substantial levels of ephrin-B1 in BPH-1 and PC3 cells (Fig. 1A). We plan to carefully compare the levels of EphB2 tyrosine phosphorylation in the BPH-1 cells grown at different levels of confluency because the presence of ephrin-B1 in these cells suggests that EphB2 should be tyrosine phosphorylated. Furthermore, EphB2 is concentrated at cell-cell

junctions in confluent cultures of BPH-1 cells (Fig. 2), suggesting that it has the ability to bind ephrin-B1 at sites of cell-cell contact. Therefore, the low EphB2 phosphorylation detected in BPH-1 cells in Fig. 1B may be due to insufficient confluency of the cells. Alternatively, ephrin-B1 may not be present on the cell surface. Immunostaining for ephrin-B1 will resolve this issue.

Unlike EphB2, the closely related EphB3 and EphB4 receptors were expressed at similar levels in BPH-1 and the prostate cancer cell lines, consistent with recently published data at the mRNA level (Fox et al., 2006). This suggests the intriguing possibility that EphB2 may have a different role in prostate cancer compared to the related EphB3 and EphB4 receptors. Indeed, recently published data indicate that EphB4 may have a tumor-promoting role in prostate cancer (Lee et al., 2005; Xia et al., 2005). These studies, however, did not examine the effects of ephrin ligand stimulation on the tumorigenic activity of EphB4. Since EphB4 is phosphorylated at very low or undetectable levels in prostate cancer cells (Fig. 1B), the described pro-oncogenic activity may occur independently of ephrin ligand stimulation.

Of the EphA receptors examined, EphA2 was similarly expressed in the prostate cell lines, except for LNCaP cells (Fig. 1A), consistent with previous reports at the mRNA level (Fox et al., 2006). EphA2 was also substantially tyrosine phosphorylated in DU145 and PC3 cells (Fig. 1B), consistent with the presence of ephrin-A ligand mRNAs in these cells (Fox et al., 2006). Nevertheless, EphA2 tyrosine phosphorylation (activation) could be further increased by stimulation with exogenous ephrin-A1 Fc ligand and also by stimulation with an agonistic peptide that we identified (Koolpe et al., 2002). Unlike the ephrin-A ligands, which are not selective for EphA2, the peptide will be useful to selectively activate EphA2 and not other EphA receptors in prostate cancer cells. EphA4 was not detected in prostate cell lines, consistent with previous reports at the mRNA level (Fox et al., 2006).

Task 2. Prepare pIRES-EGFP constructs for human EphB2 wild-type and kinase inactive mutant by subcloning and site-directed mutagenesis.

We have obtained the human EphB2 cDNA and cloned it into the pIRES-EGFP vector. However, before engineering this vector some experiments were performed by co-transfecting EphB2 in pcDNA3 together with a separate vector encoding EGFP (enhanced green fluorescent protein) rather than the bicistronic pIRES-EGFP vector.

Task 3. Determine the effects of transiently transfected EphB2 and other ligand-activated Eph receptors on DU145 prostate cancer cell growth.

In previously published experiments, it was concluded that forced expression of EphB2 in DU145 cells suppresses the growth of cultured prostate cancer cells, which lack EphB2 (Huusko et al., 2004). However, those experiments did not conclusively demonstrate an effect of EphB2 on DU145 cell growth because cells expressing EphB2 and control cells were derived from different transfections. Therefore, the observed decrease in colony growth could have resulted from differences in transfection efficiency rather than from inhibitory effects of EphB2 on prostate cancer cell growth. To overcome this potential problem, we decided to generate stably transfected clones of DU145 cells expressing EphB2. We obtained substantial expression of both wild-type and kinase dead EphB2 in polyclonal populations of cells selected with G418 for 2 weeks (Fig. 5A). The transiently transfected wild-type EphB2 was phosphorylated on tyrosine, indicating that it was activated (Fig. 5B). However, these mixed populations of cells lost EphB2 expression with continued growth in culture, even though they were kept under selection, suggesting that cells expressing low to undetectable levels of EphB2 had a growth advantage (not shown). We therefore tried to select stable clonal cell lines expressing EphB2. Even though we could isolate stable G418-resistant clones, they expressed low to undetectable levels of EphB2 (Fig. 5C), again consistent with preferential growth of clones with very low

EphB2 expression. We then transfected EphB2 in the pIRES-EGFP bicistronic vector. This allowed us to sort by FACS the cells with highest levels of EGFP and, therefore, the highest levels of EphB2 (since EphB2 was expressed from the same mRNA as EGFP). However, clones grown under selection from individual FACS-sorted cells had low to undetectable levels of EphB2 and EGFP expression, whereas we could obtain control clones with substantial EGFP expression in the absence of EphB2 (Fig. 5D).

Taken together, these results suggest that EphB2 inhibits the growth of DU145 prostate cancer cells. However, they do not provide conclusive evidence. It will therefore be necessary to express EphB2 using an inducible system, so that DU145 clones can be obtained in which EphB2 expression is induced only upon addition of doxycycline to the culture medium. This will allow a rigorous comparison of the same cells with and without EphB2 expression. We will use the "Tet-on" inducible system (Clontech), with which we have prior experience. The vectors needed to establish this system in DU145 cells are also already available in our laboratory.

The problems with maintaining expression of transfected EphB2 are likely also the reason for the inconsistent results obtained in initial experiments to assess the effects of EphB2 on 2-dimensional and 3-dimensional prostate cancer growth (Fig. 6). For these experiments, we transiently co-transfected DU145 cells with EphB2-pcDNA3 and an EGFP vector to mark the transfected cells. One day after transfection, the transfected (green fluorescent) cells were FACS sorted and equal numbers of control and EphB2-transfected cells were plated in cell culture dishes. In some experiments, the cells were counted after 4 days of growth in tissue culture plates (Fig. 6A). We obtained inconsistent results, with only some experiments showing effects of EphB2. The sorted cells were also used to generate 3-dimensional spheroids grown in hanging drops (Kelm et al., 2003), which better mimic the 3-dimensional growth of tumor tissue. The spheroids were photographed after 5 days of growth (Fig. 6B). In two separate experiments, we did not observe differences between EphB2-transfected and vector control-transfected spheroids. In these experiments we could not verify the levels of EphB2 expression by immunoblotting due to the small number of cells in the spheroids. However, these experiments will be repeated once we obtained clones of DU145 cells that inducibly express EphB2.

Aim 2. Characterize the effects of EphB2 signaling pathways on prostate cancer cell survival, proliferation, migration and invasion

The problems encountered in Aim 1 in generating stably transfected DU145 cells with high levels of EphB2 expression indicate that an inducible system where EphB2 expression can be regulated with doxycycline will be much more suitable to unequivocally characterize the effects of EphB2 signaling pathways in prostate cancer cells. Therefore, we will use this approach for the experiments proposed in this aim.

Aim 3. Assess the functional effects of EphB2 mutations identified in prostate cancer specimens

These experiments are planned for the third year. However, we have already obtained a vector encoding the human kinase dead EphB2 mutant, which will be used to model the effects of mutations that inactivate EphB2 catalytic activity.

Aim 4. Determine whether inactivation of the EphB2 gene accelerates tumorigenesis in a transgenic mouse model of prostate cancer

In the application, we proposed to use the Pten;PB-Cre mouse model of prostate cancer in combination with EphB2 gene inactivation to examine the role of EphB2 in prostate cancer

progression. In this model, conditional inactivation of the Pten phosphatase gene in the prostate epithelium has been reported to cause prostatic intraepithelial neoplastic (PIN) lesions that develop to metastatic adenocarcinoma (Wang et al., 2003). On the advice of the reviewers, we have obtained PIN tissue from the Pten;PB-Cre transgenic mice and examined EphB2 expression compared to matched normal prostate tissue (Fig. 7). This confirmed that EphB2 is expressed in mouse prostate epithelium and showed that EphB2 expression does not change during the initial phases of prostate cancer development. In another mouse prostate cancer model, driven by the Myc oncogene, EphB2 levels also were found to be the same in the normal epithelium and in prostate cancer tissue ((Ellwood-Yen et al., 2003) supplementary information). These results indicate that EphB2 expression is not downregulated during prostate cancer progression in the available mouse models as it appears to be in a fraction of human prostate cancers (Huusko et al., 2004) and in prostate cancer cell lines (Fig. 1).

Recent work has also shown that the Pten;PB-Cre model does not exhibit as fast and reliable tumor progression as initially described. First, a recent report has shown that Pten-deficient tumorigenesis in the prostate is suppressed by p53-dependent cellular senescence, which accounts for a slow tumor development and low degree of tumor malignancy in the Pten;PB-Cre mice (Chen et al., 2005). Second, with support from DOD grant number W81XWH-04-1-0888 our collaborator Dr. Robert Oshima also found that the progression of the Pten;PB-Cre tumors was much slower than expected based on the previously published information and did not reach invasive and metastatic stages (personal communication). By laser-capture microdissection of PIN lesions from the Pten;PB-Cre mice, Dr. Oshima also found that Cre-mediated recombination in epithelial cells was rather incomplete (Fig. 8). Thus, PIN lesions likely represent a combination of cells with recombined Pten alleles and cells without recombined alleles or with only one recombined allele. Inactivation of the Pten gene is therefore not as complete as expected from previously published results with the PBcre4 transgene, and this likely also contributes to the slow tumor progression. Whether the effectiveness of Cre recombination may to depend on the background of the mice remains to be determined. Third, the reviewers expressed concern with regard to possible crosstalk between EphB2 and the activated Akt pathway in the Pten;PB-Cre mice.

The reasons outlined above, and the complicated crosses required to obtain the appropriate genotypes, suggest that an alternative in vivo model would be advantageous for our experiments. In the application, we had outlined two alternative in vivo models to understand the role of the EphB2 receptor in human prostate cancer progression if the Pten;PB-Cre proved unsuitable. One model involves using the Myc transgenic model of prostate cancer in combination with EphB2 gene inactivation (Ellwood-Yen et al., 2003). The other model involves generating tumor xenografts in nude mice from stably transfected DU145 cells. Our preliminary results described above indicate that we should use the DU145 cells in which EphB2 expression can be restored in an inducible manner, which will be generated for the studies proposed in Aim 1. The approach of choice will become more evident after generation and characterization of the inducible cell lines.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that EphB2 is expressed at higher levels in non-transformed prostate epithelial BPH-1 cells than in prostate cancer cell lines.
- Determined that EphB2 is localized at cell-cell junctions in non-transformed BPH-1 prostate epithelial cells grown to confluency.
- Determined that EphB2 is tyrosine phosphorylated (activated) at low to undetectable levels in prostate cancer cells.

- Determined that EphB2 protein is expressed at similar levels in normal mouse prostate epithelium and in intraepithelial prostatic neoplasia (PIN) that develops in mice in which the Pten gene is conditionally inactivated in prostate epithelium.
- Compared expression levels of several Eph receptors and ephrins in non-transformed BPH-1 prostate epithelial cells and in prostate cancer cell lines.
- Compared tyrosine phosphorylation levels of several Eph receptors in non-transformed prostate epithelial BPH-1 cells and in prostate cancer cell lines.
- Established that DU145 cells transfected with different plasmids encoding EphB2 cDNA initially express high levels of tyrosine phosphorylated EphB2, but rapidly lose expression.
- Compared the growth of populations of DU145 cells transiently transfected with EphB2 or control vector, marked with EGFP and isolated by FACS sorting. Obtained inconsistent results, presumably due to variable and changing levels of EphB2 expression and activation following transient transfection.
- Found that the EphB4 receptor expressed in DU145 prostate cancer cells can be stimulated with the ephrin-B1 ligand. This result is different from previous reports in the literature, which indicated that EphB2 can only be activated by the ephrin-B2 ligand.
- Found that although the EphA2 receptor is highly phosphorylated (activated) in PC3 prostate epithelial cells, phosphorylation is not maximal and treatment with ephrin-A1 substantially enhances EphA2 phosphorylation. Thus, it will be possible to examine the effects of increased EphA2 activation in these cells.

REPORTABLE OUTCOMES

Constructs:

1. pcDNA3 encoding the long isoform of human wild-type EphB2 (subcloned from a different vector obtained from Dr. Mitsutoshi Nakada at TGen).
2. pcDNA3 encoding the long isoform of human kinase dead EphB2 (subcloned from a different vector obtained from Dr. Mitsutoshi Nakada at TGen).
3. pcDNA3 encoding the short isoform of human wild-type EphB2 (generated by mutagenesis of the long isoform). This is the most widely expressed isoform.
4. pIRES-EGFP encoding the short isoform of human wild-type EphB2.

Cell lines:

1. DU145 stable clones expressing control EGFP.
2. DU145 stable clones expressing low levels of human wild-type EphB2.

CONCLUSION

We have found that multiple Eph receptors are expressed in prostate cancer cells. We detected EphB2 at higher levels in a non-transformed prostate epithelial cell line compared to several prostate cancer cell lines, consistent with the idea that loss of EphB2 may aid cancer progression. In addition, we found that prostate cancer cells transfected with expression vectors encoding EphB2 initially expressed high levels of activated EphB2 but then lost expression,

suggesting that EphB2 expression is detrimental to the growth of the cells. However, these experiments did not produce conclusive results because constant EphB2 expression could not be maintained for prolonged periods. Therefore, it will be important to establish an inducible system for regulating EphB2 expression in prostate cancer cell lines in a predictable manner.

Recent results from several laboratories have indicated that genetically engineered mice in which prostate tumors are driven by Pten gene inactivation may not be ideal to study the role of EphB2 in prostate cancer. Prostate cancer xenografts grown in nude mice using cells with inducible EphB2 expression or the Myc transgenic mouse model of prostate cancer appear to be more suitable.

Our data and reports that have appeared in the literature during the past year support the notion that Eph receptors play a role in prostate cancer. The role of EphB2 appears to be as a tumor suppressor, whereas other Eph receptors such as EphB4 may have a different influence on prostate cancer progression. Clearly this is an important area of investigation that will help understand the pathogenesis of prostate cancer. The information obtained from these studies will also help guide the design of appropriate treatment strategies and determine whether prostate cancers should be screened for Eph receptor and ephrin mutations for prognostic purposes.

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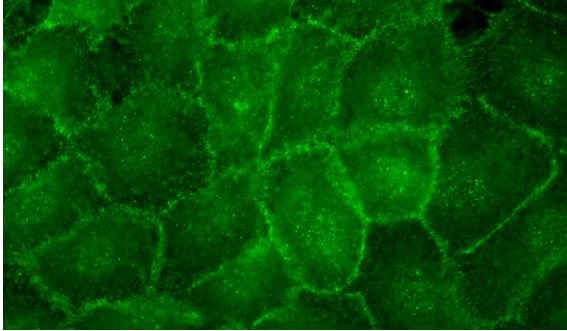


Figure 2. EphB2 is localized at cell-cell junctions in confluent BPH-1 non-transformed prostate epithelial cells. Cells were grown on glass coverslips, fixed with methanol and labeled with anti-EphB2 antibodies followed by a green fluorescent secondary antibody. EphB2 immunoreactivity outlines cell-cell junctions.

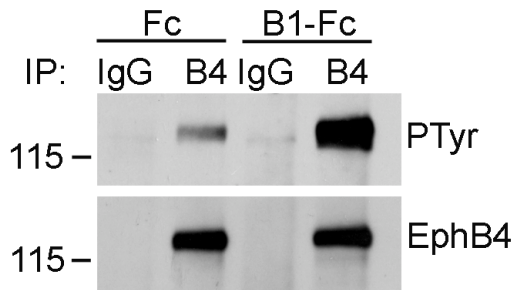


Figure 3. Ephrin-B1 Fc stimulation increases EphB4 tyrosine phosphorylation (activation) in DU145 prostate cancer cells. DU145 cells were starved overnight and stimulated for 10 min with 1 μ g/ml ephrin-B1 Fc or Fc control preclustered with anti-Fc antibodies. Immunoprecipitated EphB4 was probed by immunoblotting with anti-phosphotyrosine antibodies (PTyr) and reprobed with anti-EphB4 antibodies. IgG, control immunoprecipitates with non-immune antibodies. EphB4 phosphorylation was substantially increased by ephrin-B1 Fc.

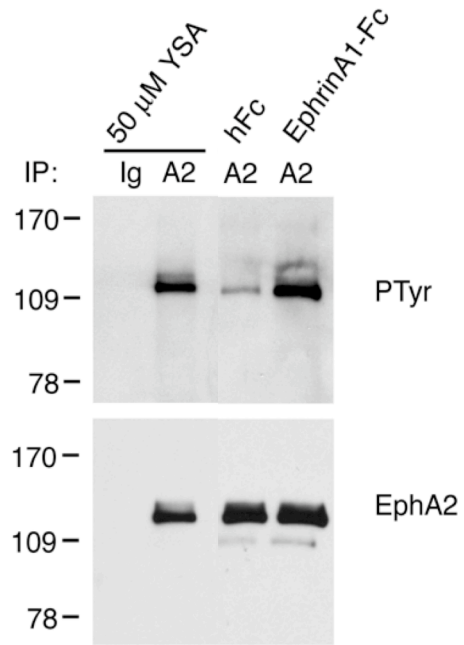


Figure 4. EphA2 tyrosine phosphorylation in PC3 prostate cancer cells is increased by ephrin-A1 Fc and the agonistic YSA peptide. PC3 cells were starved overnight and stimulated for 15 min with 0.1 μ g/ml ephrin-A1 Fc, 2 μ g/ml Fc control and 50 μ M biotinylated YSA peptide. Immunoprecipitated EphA2 was probed by immunoblotting with anti-phosphotyrosine antibodies (PTyr) and reprobed with anti-EphA2 antibodies. Ig, control immunoprecipitates with non-immune antibodies. Although EphA2 is constitutively phosphorylated on tyrosine residues in PC3 cells, phosphorylation can be substantially increased even with the low concentration of ephrin-A1 Fc used or with the YSA peptide.

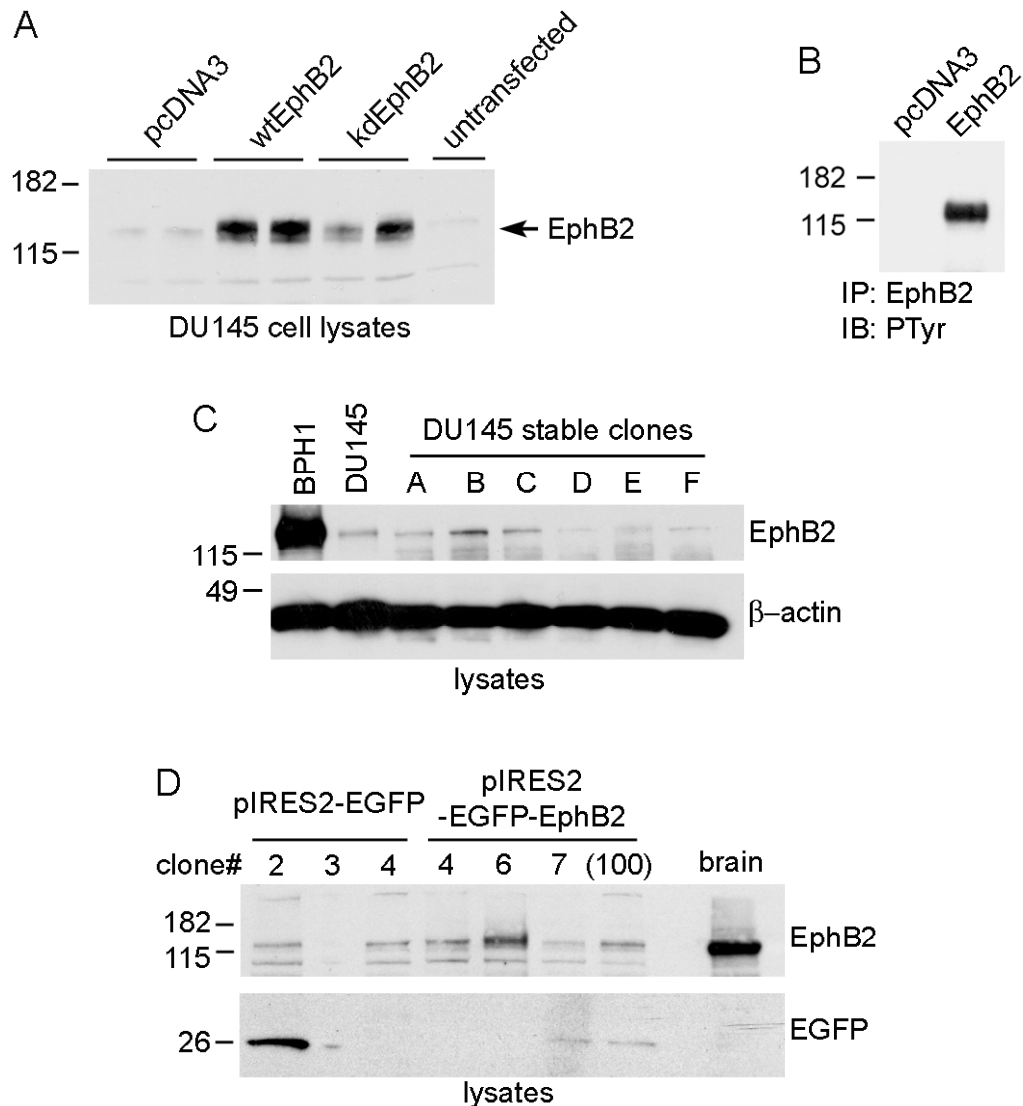


Figure 5. Transfection of DU145 prostate cancer cells with EphB2. **(A)** Lysates from polyclonal populations of DU145 cells transfected with wild-type EphB2 (wtEphB2), kinase dead EphB2 (kdEphB2) or empty vector as a control (pcDNA3) and grown under G418 selection for 2 weeks were probed by immunoblotting with anti-EphB2 antibodies. Samples from two different cultures are shown for each transfection. Untransfected DU145 cells were used as an additional negative control. Both catalytically active and inactive EphB2 remain overexpressed after 2 weeks. **(B)** DU145 cells transiently transfected for 48 hours with wild-type EphB2 or pcDNA3 as a control were immunoprecipitated (IP) with anti-EphB2 antibodies. The immunoprecipitates were then probed by immunoblotting (IB) with anti-phosphotyrosine antibodies (PTyr), showing that transiently transfected EphB2 is tyrosine phosphorylated (activated). **(C)** EphB2 expression is undetectable over background in stably transfected clones expressing wild-type EphB2 (at the long exposures used, the EphB2 antibody labels a background band at the position of EphB2). EphB2 levels in the BPH-1 non-transformed prostate epithelial cells are shown for comparison. Probing with anti-β-actin antibodies verifies equal amounts of protein in the lysates. **(D)** DU145 cells were transfected with EphB2 in the pIRES2-EGFP bicistronic vector (from Clontech) or with the vector alone as a control, selected with G418 for a day, FACS sorted based on EGFP green fluorescence, plated individually into 96-well plates and grown for 3 weeks before probing by immunoblotting for EphB2 and EGFP expression. Representative clones are shown. Some clones transfected with the control vector maintained high EGFP expression, while clones transfected with EphB2 and EGFP had low EphB2 and EGFP expression. Clone 6 has EphB2 expression above background but undetectable EGFP. In some samples, EGFP but not EphB2 is detectable. (100) indicates a polyclonal population of cells kept under selection in parallel with the clones, as a control.

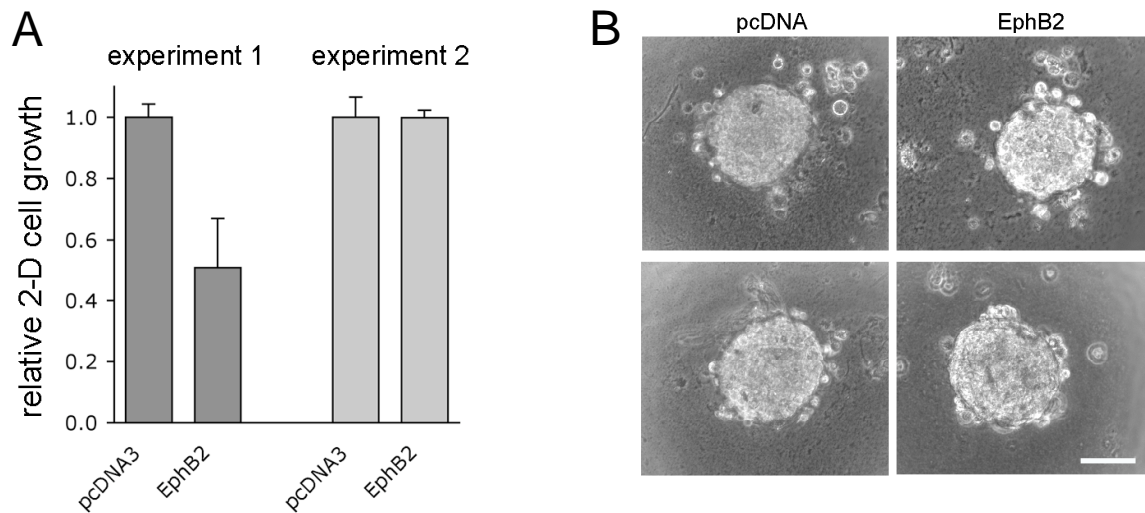


Figure 6. Effects of EphB2 transfection on the growth of DU145 prostate cancer cells. DU145 cells were transfected with pcDNA3 control vector together with EGFP vector or with EphB2-pcDNA3 together with the EGFP vector. After 1 day, the transfected cells (green fluorescent) were sorted by FACS and plated in tissue culture plates. In **(A)** the cells were counted 4 days later. The results from 2 experiments in which the cells were counted in triplicate are shown (averages \pm SD). While the results of the first experiments suggest that EphB2 expression inhibits cell growth, this result was not reproducible, as shown in the second experiment. In **(B)** the cells were allowed to recover in 2-D culture for 2 days and then used to generate 3-D spheroids in hanging drops of medium. Spheroids were allowed to grow for 5 days before photographs were taken. Spheroids made from cells expressing EphB2 were indistinguishable from control spheroids in size and appearance.

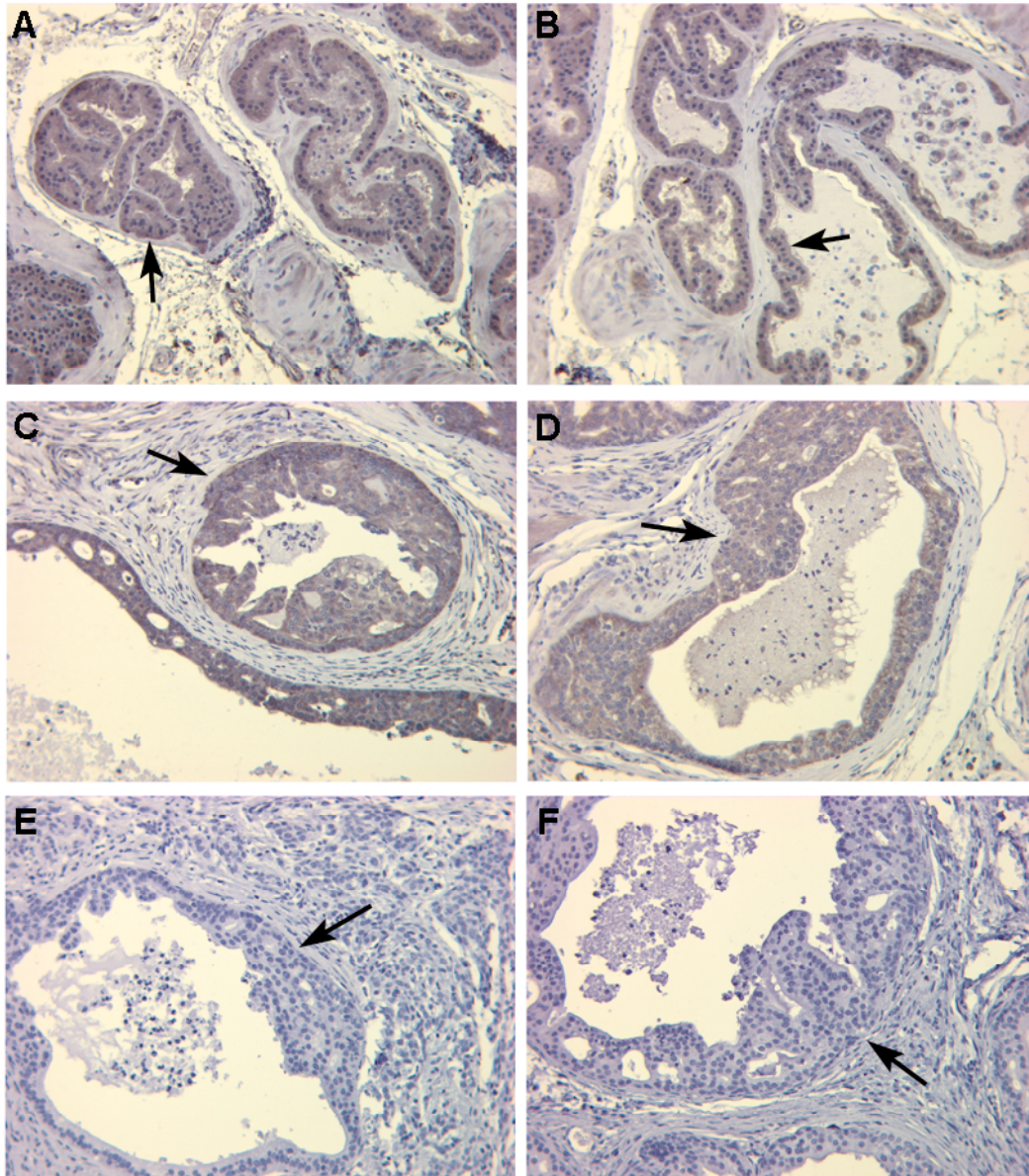


Figure 7. EphB2 is expressed at similar levels in mouse prostate gland epithelium and prostatic intraepithelial neoplasia (PIN). Immunostaining of mouse prostate frozen sections with anti-EphB2 antibodies (in brown). The sections were counterstained with hematoxylin, in blue. **(A, B)** Mouse normal prostate in *Pten^{flox/+}* mice. **(C,D)** Prostatic intraepithelial neoplasia in *Pten^{flox/flox}, PB-Cre* mice. **(E, F)** Sections from the same prostate as in C and D, but incubated with secondary antibody only (negative control). Arrows mark the epithelium.

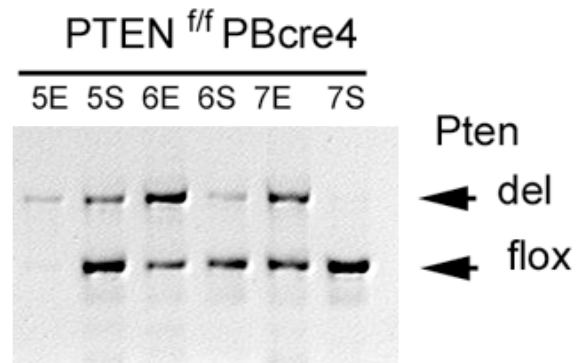


Figure 8. Cre-mediated inactivation of the Pten gene in the epithelium of Pten;PB-Cre mice is incomplete. Epithelial and stromal cells from PIN lesions were isolated from prostate tissue sections by laser-capture microdissection. Pten PCR products of DNA isolated from dissected material were separated on an agarose gel. The migration position of the PCR products for the Cre deleted (del) and undeleted flox alleles are shown on the right. Animal numbers and the type of tissue (E, epithelial or S, stroma) are indicated at the top. The results show that recombination is confined to the epithelial tissue (the slight signal for the Cre deleted band in lanes 5S and 6S is likely due to epithelial contamination). However, recombination in the epithelial tissue is much less than expected from previously published data, suggesting that many of the prostate epithelial cells still express Pten.